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(54) Title: PROCESS FOR LYOPHILIZING CELLS, CELL-LIKE MATERIALS AND PLATELETS IN A MIXTURE OF BIOCOMPATIBLE AMPHIPATHIC POLYMERS

(57) Abstract

A process and medium are disclosed for the lyophilization of cells (including platelets) which comprises the use of solutions including comprising monosaccharide hexoses and pentoses, and a mixture of at least two biocompatible amphipathic polymers.

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# PROCESS FOR LYOPHILIZING CELLS, CELL-LIKE MATERIALS AND PLATELETS IN A MIXTURE OF BIOCOMPATIBLE AMPHIPATHIC POLYMERS

## FIELD OF THE INVENTION

This invention relates to the general field of biochemistry and medical sciences, and specifically to processes for the preservation, storage and reconstitution of cells, particularly red blood cells and platelets, and cell-like materials (such as hemosomes).

## BACKGROUND AND SUMMARY OF THE INVENTION

Laboratory cell preservation and storage have been significant problems for a variety of plant and animal cells. Freezing the cells in an aqueous solution and thawing the cells prior to use is not uncommon, but the viability of the cells after this process can be affected. In addition, the expense of keeping the cells frozen is significant, especially when liquid nitrogen is used to maintain the frozen cells at -196°C. Liquid nitrogen storage is cumbersome when large numbers of frozen samples or cell culture lineages have to be maintained.

For example, there has been a need for improved methods for the storage of blood and blood constituents. The predominant role for delivery of oxygen from the lungs to peripheral tissues is carried out by erythrocytes, i.e., red blood cells (RBC). The oxygen is furnished from the lungs by an exchange-diffusion system brought about by a red, iron-containing protein called hemoglobin which comprises most of the total cell protein in a mature red cell. When hemoglobin combines with oxygen, oxyhemoglobin is formed and after oxygen is given up to the tissues, the oxyhemoglobin is reduced to deoxyhemoglobin.

The red cell membrane is composed of two major structural units, the membrane bilayer and a cytoskeleton. A lipid bilayer and integral membrane proteins form the membrane bilayer, which has little structural strength and fragments readily by vesiculation. The other major component, the membrane skeleton, stabilizes the membrane bilayer and provides resistance to deformation. The cytoskeleton is linked to the bilayer in the erythrocyte membrane, possibly by lipid-protein as well as protein-protein associations. The hemoglobin, and other RBC components, are contained within the red cell membrane.

In adults, bone marrow is active in the formation of new red blood cells. Once new erythrocytes enter the blood, these cells have an average lifetime of about 120 days. In an average person, about 0.83% of the erythrocytes are destroyed each day by phagocytosis, hemolysis or mechanical damage in the body, and the depleted cells are renewed from the bone marrow.

A wide variety of injuries and medical procedures require the transfusion of whole blood or a variety of blood components. Every patient does not require whole blood and, in fact, the presence of all of the blood components can cause medical problems. Separate blood fractions can be stored under those special conditions best suited to assure their biological activity at the time of transfusion. For example, when donor blood is received at a processing center, erythrocytes are separated and stored by various methods. Such cells are storable in citrate-phosphate-dextrose at 4°C for up to five weeks, generally as a unit of packed erythrocytes having a volume of from 200 to 300 ml and a hematocrit value (expressed as corpuscular volume percent) of 70 to 90. Erythrocytes may also be treated with glycerol and then frozen at from -30° to -196°C and stored for up to seven years in a glycerol solution, but must be kept frozen at low temperatures in order to survive sufficiently for transfusion. Both these methods require careful maintenance of storage temperature to avoid disruption of the desired biological activity of the erythrocytes. Current practice involves frozen storage of packed red cells in 40% w/v glycerol in -80°C mechanical freezers. The thawed cells must be washed extensively with sterile saline to remove the glycerol prior to transfusion. This glycerol freeze-thaw method provides a twenty-four hour survival time for at least 70% of the transfused cells, which is considered to be an acceptable level for use in transfusion practice in accordance with the American Association of Blood Bank standards.

It has thus been a desideratum to obtain a method for the storage of cells, and in particular red blood

cells, which is not dependent on the maintenance of specific storage temperatures or other storage conditions. Such a method would facilitate the availability of erythrocytes and platelets for medical purposes and assist in the storage and shipment of various mammalian cells and plant cells, particularly protoplasts, for research and hybrid cell culture development.

One such desired method has been the lyophilization (freeze-drying) of cells, since such cells could be stored at room temperature for an extended period of time and easily reconstituted for use. Freeze-dried cells (such as erythrocytes, platelets, or cell-like material, such as, hemosomes) could thus be easily stored for use in transfusions. However, prior to our invention, it has been not practically feasible to freeze-dry cells in a manner which permits the reconstitution of the cells, in the case of erythrocytes, to form erythrocytes with an intact cell membrane, cytoskeleton and biologically-active hemoglobin, i.e., viable red blood cells. When RBCs have been lyophilized according to previous methods, for example in either an aqueous or phosphate-buffered saline (PBS) solution, the reconstituted cells are damaged to the extent that the cells are not capable of metabolizing, or the cell hemoglobin cannot carry oxygen or the cells lyse upon rehydration and are not useful for transfusion. Glutaraldehyde-fixed erythrocytes, which have been lyophilized and reconstituted, have found use primarily in agglutination assays, in which only the preservation of certain cell surface antigens is desired. These fixed cells are metabolically non-

viable and are unsuitable for use in transfusion medicine.

The process of the present invention allows for the lyophilization of red blood cells or platelets under conditions which are not deleterious to the structure and the biological activity of the cell, and which permits the reconstitution of the lyophilized red blood cells or platelets to form cells in which the biological activity found in freshly collected cells is preserved at useful levels. The cells may be from in vitro cultures, peripheral blood cells, blood stem cells, or cell-like materials, such as liposomes, hemosomes or cell membrane ghosts. Furthermore, these may be mammalian cells, hybridoma cells, or any other type of cell.

Briefly, the process comprises immersing a plurality of cells in an essentially isotonic aqueous solution containing a carbohydrate, and a mixture of at least two types of amphipathic polymers, freezing the solution, and drying the solution to yield freeze-dried cells which, when reconstituted, produce a significant percentage of intact and viable cells.

While the invention is applicable to a wide variety of plant and animal cells, the process of the invention is preferably applied to red blood cells or platelets and allows for the lyophilization under conditions which maintain structure of the cell and the biological activity of the hemoglobin, and which permits the reconstitution of the lyophilized red blood cells or platelets to allow use on a therapeutic level. The carbohydrate of the invention is biologically compatible with the cells, that is,

non-toxic and non-disruptive to the cells, and is preferably one which permeates, or is capable of permeating, the membrane of the cells. Such membrane-permeant carbohydrates apparently protect the intracellular components, to include the oxyhemoglobin, from freezing and drying damage.

The carbohydrate may be selected from the group consisting of monosaccharides, since disaccharides do not appear to permeate the membrane to any significant extent. Monosaccharide pentoses and hexoses are preferred in concentrations of from about 7.0 to 37.5%, preferably about 23%. Xylose, glucose, ribose, mannose and fructose are employed to particular advantage.

The use of a mixture of water soluble, biologically compatible amphipathic polymers in addition to the carbohydrate adds significantly to the percentage of biologically-active hemoglobin (in the case of red blood cells) which is retained in the cells and recovered after reconstitution of red blood cells after lyophilization. Retention of cell hemoglobin provides an easy assay for cell lysis or leakiness; use of polymers in the present invention appears to minimize loss of cell hemoglobin and therefore preserves cell integrity. The polymers will preferably be amphipathic, meaning that there are hydrophilic and hydrophobic portions on a single molecule of the polymer. The mixture of polymers may be present in the buffered lyophilization solution in total concentrations of from 0.7% (by weight) up to saturation. Preferably, each of the polymer types in the mixture has a molecular weight in the range of from about 1K to about 600K (number average molecular

weight). Preferably, at least one of the types of polymers of the mixture will preferably have a molecular weight from about 5K to 400K, and most preferably from 20K to 360K. Also, one of the types of polymers of the mixture will preferably have a molecular weight in the range of about 100K to about 600K, most preferably in the range of about 100-500K. For a mixture of two different polymer types, each of the polymer types may be present in a concentration of from about .35% (by weight) up to its limit of solubility in the buffered lyophilization solution. Polymers selected from the group consisting of polyvinylpyrrolidone (PVP), polyvinylpyrrolidone derivatives, dextran, dextran derivatives, amino acid based polymers (i.e., proteins) and hydroxyethyl starch (HES) may be employed. Other amphipathic polymers may be used, such as poloxamers in any of their various forms. In a preferred embodiment, a mixture of PVP (molecular weight in the range of about 20K-360K) and HES (molecular weight in the range of about 100K-500K) is employed in the buffered lyophilization solution.

The use of the carbohydrate-polymer solution in the lyophilization of red blood cells allows for the recovery of intact cells, a significant percentage of which contain biologically-active hemoglobin. While not intending to be bound by any theory, the amphipathic properties of the polymer allow them to bind to the cell membrane while protecting the membrane surface by extension of the hydrophilic portion into the aqueous environment. This may alleviate the damage to the cell membrane which causes other problems, such as cell aggregation.

In addition, the lyophilization buffer as well as the reconstitution buffer or washing buffer may further contain certain supplements which are particularly useful if the cells are cellular blood matter, including red cells, platelets, lymphocytes, stem cells; or other cell-like materials such as liposomes, hemosomes or membrane ghosts. While not intending to be limited by theory, it is believed that the supplements fall into three categories which serve to enhance the lyophilization, reconstitution or washing processes in certain ways. One class of supplements comprises antioxidants such as glutathione or alpha-tocopherol. It is believed that such antioxidants assist a cell in reducing oxidation damage (such as by cell membrane lipid peroxidation) which may otherwise occur during lyophilization or reconstitution. A second class of supplements comprises chelating agents such as EDTA or desferrioxamine, which have the ability to scavenge free iron released from the degradation of cellular hemoglobin. The free iron or hemichromes are detrimental since they may in turn catalyze oxidative damage to cells. A third class of supplements comprises amino acid based polymers (i.e., peptides and proteins), such as serum albumin which may act as a coating agent to coat the surface of the cells, thereby minimizing the formation of cell-cell aggregates.

In particular, preferred supplements include glutathione (GSH) preferably in a concentration of 1-60 mM in the buffer (either lyophilization, reconstitution or wash buffer); alpha-tocopherol, preferably in the concentration of 1-3 mg/gm RBC; EDTA in a preferred concentration of 1-10 mM;

desferrioxamine in a concentration of 1-10 mM; and albumin in a concentration of 0.5-14% (w/v). Either human or bovine serum albumins are preferred.

As is shown by the embodiments set forth below, the described solutions provide media which permit cells, particularly red blood cells, to be subjected to the stresses of freezing, water sublimation and reconstitution and to form freeze-dried cells which may be reconstituted to yield cells which are capable of functioning normally.

Unless indicated otherwise by the terminology or the context, all percentages set forth herein are expressed as weight/volume percentages (i.e., weight of the solute versus the total volume of the solution).

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph of the methemoglobin half-life in samples of reconstituted lyophilized RBCs according to the invention and non-lyophilized RBCs.

FIG. 2 is a graph of the linear regression of methemoglobin over time in reconstituted lyophilized RBCs according to the invention and non-lyophilized RBCs.

## DESCRIPTION OF THE PREFERRED EMBODIMENT

As noted above, the process of the invention provides media for the lyophilization of erythrocytes.

The term lyophilization is broadly defined as freezing a substance and then reducing the concentration of one of the solvents, namely water, by sublimation and desorption, to levels which will no longer support biological or chemical reactions. Usually, the drying step is accomplished in a high vacuum. However, with respect to the storage of cells and particularly erythrocytes, the extent of drying (the amount of residual moisture) is of critical importance in the ability of cells to withstand long-term storage at room temperature. In the method of the invention, cells may be lyophilized to a residual water content of less than 10%, preferably less than 5%, and most preferably to a water content of less than 3%.

The buffered lyophilization solution may contain, in addition to the monosaccharide and amphipathic polymer mixture, adjuvants, buffering agents, salts, cofactors, and the like. A particularly preferred lyophilization buffer contains the following components:

| | | | |
|---|---|---|---|
| 10.0 | mM | Glutathione (reduced) | 3.07 g/l |
| 10.0 | mM | Inosine | 2.68 g/l |
| 5.0 | mM | Adenine | 0.69 g/l |
| 0.75 | mM | Nicotinic acid | 0.09 g/l |
| 0.75 | mM | Glutamine | 0.11 g/l |
| 0.49 | mM | $MgCl_2 \cdot 6H_2O$ | 0.10 g/l |
| 1.47 | mM | $KH_2PO4$ | 0.20 g/l |
| 8.1 | mM | $Na_2HPO_4 \cdot 7H_2O$ | 2.17 g/l |
| 1.7 | M | Dextrose | 306.3 g/l |
| 3.0 | wt/v % | PVP(MW360K) | 30.0 g/l |
| 15.0 | wt/v % | M-HES(MW 500K) | 150.0 g/l |

In a typical lyophilization procedure, whole blood or packed red blood cells are washed on a COBE 2991 cell washer with dextrose saline by an automated protocol

designed to yield a leukocyte-free packed red cell suspension.

The cells are mixed with lyophilization buffer at a hematocrit of 30%-40%.

The lyophilization buffer is as described above, with the polymer mixture used in each test set forth in Table 1. As a control, one run was performed using only 20% 24K PVP as the polymer.

The sample is then placed on a conventional pharmaceutical shelf freeze-dryer and the samples are then frozen on the refrigerated shelf, then vacuum is applied and the sample is allowed to dry until the sample is thoroughly dried as determined by a 58% weight loss.

To reconstitute the dried samples, an equal volume of pre-warmed reconstitution buffer at 37°C is added to samples and agitated until sample is fully hydrated. Preferably the reconstitution buffer will contain a polymer as described above in connection with the lyophilization buffer (concentration preferably in the range of about 1-20 wt. %) which is amphipathic having a MW in the range of 1-600K, preferably 1-360K.

A preferred reconstitution buffer is as follows:

| | | | |
|---|---|---|---|
| 5.0 | mM ATP | 2.76 | g/l |
| 1.47 | mM $KH_2PO_4$ | 0.20 | g/l |
| 8.1 | mM $Na_2HPO_4 \cdot 7H_2O$ | 2.17 | g/l |
| 19.0% | 10K PVP | 190.0 | g/l |

For the test, reconstituted sample is prediluted with an equal volume of reconstitution buffer and agitated until thoroughly mixed. The reconstituted and prediluted cells are centrifuged at room temperature.

Another reconstitution buffer is as follows:

| | | |
|---|---|---|
| 2.0 | mM KCl | 0.15 g/l |
| 1.47 | mM $KH_2PO_4$ | 0.20 g/l |
| 100.7 | mM NaCl | 6.47 g/l |
| 8.1 | mM $Na_2HPO_4$ | 1.15 g/l |
| 19.0% | 24K PVP | 190.0 g/l |

The reconstituted sample is prediluted with an equal volume of reconstitution buffer and swirled until thoroughly mixed. At this point the cell suspension can be aseptically transferred to a sterile, enclosed cell washing system such as the COBE model 2991 cell washer. The reconstituted and prediluted cells are centrifuged at room temperature to collect the cells.

The pellet is resuspended in wash buffer and centrifuged. The wash buffer will preferably contain a polymer as described above in connection with the lyophilization buffer (concentration preferably in the range of about 1-20 wt/v %) which is amphipathic having a MW in the range of 1-600K, preferably 1-360K.

The preferred wash buffer is as follows:

| | | |
|---|---|---|
| 10.0 | mM Inosine | 2.68 g/l |
| 5.0 | mM Adenine | 0.69 g/l |
| 0.75 | mM Nicotinic acid | 0.09 g/l |
| 0.75 | mM Glutamine | 0.11 g/l |
| 0.49 | mM $MgCl_2$ $6H_2O$ | 0.10 g/l |
| 30.0 | mM KCl | 2.24 g/l |
| 30.0 | mM NaCl | 1.75 g/l |
| 10.0 | mM $Na_2HPO_4 \cdot 7H_2O$ | 2.68 g/l |

| | | |
|---|---|---|
| 20.0 | mM Glucose | 3.60 g/l |
| 16.0% | 40K PVP | 160.0 g/l |

Another wash buffer is as follows:

| | | |
|---|---|---|
| 10.0 | mM Inosine | 0.15 g/l |
| 5.0 | mM Adenine | 0.69 g/l |
| 0.75 | mM Nicotinic acid | 0.09 g/l |
| 0.75 | mM Glutamine | 0.11 g/l |
| 0.49 | $MgCl_2$ $6H_2O$ | 0.10 g/l |
| 5.0 | KCl | 0.37 g/l |
| 75.0 | mM NaCl | 4.40 g/l |
| 10.3 | mM $Na_2HPO_4$ | 1.46 g/l |
| 20.0 | mM Glucose | 3.60 g/l |
| 16.0% | 24K PVP | 160.0 g/l |

An optional step involves a diluent buffer step to eliminate any fragile cells. The pellet is resuspended in a diluent buffer at a 10-50 fold dilution and centrifuged.

The preferred diluent buffer is as follows:

| | | |
|---|---|---|
| 129.5 | mM NaCl | 7.57 g/l |
| 5.0 | mM $Na_2HPO_4 \cdot 7H_2O$ | 1.34 g/l |

Another diluent buffer is as follows:

| | | |
|---|---|---|
| 61.1 | mM Sodium Pyrophosphate | 16.23 g/l |
| 1.19 | mM KCl | 0.15 g/l |
| 0.88 | mM $KH_2PO_4$ | 0.12 g/l |
| 11.1 | mM NaCl | 0.65 g/l |
| 4.86 | mM $Na_2HPO_4$ | 0.69 g/l |
| 8.89 | mM ATP | 4.9 g/l |

The pellet is resuspended in the final solution, transfusion buffer, and centrifuged. This step is repeated once. The transfusion buffer will preferably contain a polymer as described above in connection with the lyophilization buffer (concentration preferably in the range of about 1-20 weight/v %) which is amphipathic having a MW in the range of 1-600K, preferably 1-10K.

The preferred transfusion buffer is as follows:

| | | |
|---|---|---|
| 77.0 | mM NaCl | 4.50 g/l |
| 5.0 | mM $Na_2HPO_4 \cdot 7H_2O$ | 1.34 g/l |
| 10.0 | mM Glucose | 1.80 g/l |
| 10.0% | 2.5K PVP | 100.0 g/l |

Another transfusion buffer is as follows:

| | | |
|---|---|---|
| 68.4 | mM NaCl | 4.00 g/l |
| 5.0 | mM $Na_2HPO_4$ | 0.71 g/l |
| 10.0 | mM Glucose | 1.80 g/l |
| 10.0% | 2.5K PVP | 100.0 g/l |

To determine the hemoglobin recovery a 200 uL sample of cells is centrifuged for 5 min. at 5000 rpm. The pellet and supernatant are separated and 180 uL of water is added to the pellet, which is lysed by vortexing. To each sample 1 mL of Drabkins reagent is added, and after standing at R.T for 15 min. the absorbance at 540 nm. Recovery = $A_{540}$ pellet/$A_{540}$ pellet + $A_{540}$ supernatant.

To determine whole blood stability of reconstituted cells, $^{51}Cr$ as sodium chromate in a 1 mCi/ml sterile NaCl solution is added to a sample of reconstituted cells. 5$\mu$Ci of $^{51}Cr$ is added for every 0.1 ml of packed RBC pellet. The labelled pellet is incubated 15 min. at 37°C after which the labelling reaction is stopped by addition of 1 ul of ascorbic acid (50mg/ml in buffer) to every 0.1 ml of pellet. The pellet is then allowed to incubate another 5 min. at room temperature. The labelled sample is then washed 2 to 3 times in transfusion buffer. An aliquot of labelled cells is then transferred to 5 ml of autologous whole blood and the stability determined

by the lysis of labelled cells at time points up to 24 hours.

The amount of free $^{51}Cr$ in the supernatant after centrifuging indicates the amount of cell lysis. For convenience, a 4-hour incubation is used, since lysis (if any) is complete by then.

Cell stability data (using the $^{51}Cr$ tracer) show the stability and integrity of the lyophilized, constituted red blood cells. The $^{51}Cr$ binds to the internal cell hemoglobin, and is released into the assay supernatant (therefore, lost) if the cells lyse. Thus, retention of $^{51}Cr$ in the pellet measures cell integrity. The high cell stability indicates sufficient cell preservation to be useful for diagnostic use, or for use in transfusion medicine.

The following examples are provided by way of illustration.

## EXAMPLE 1

Lyophilized reconstituted human red cells tested using the above procedures. Red cells were lyophilized using one polymer or a polymer mixture, and the whole blood stability of $^{51}Cr$ labeled reconstituted cells was studied. The reconstituted cells were processed using an automated cell washer as described in Example 2. The results are described as follows (Table I):

TABLE 1

| Lyophilization Buffer Polymer Composition | Hemoglobin Recovery | Mean Cellular Volume | 4 hr. Whole Blood Stability |
|---|---|---|---|
| 20% 24K PVP (Control) | 24.3 ± 2.2 | 87.6 ± 6.2 fl | 50.5 ± 15.5% |
| 5% 24K PVP 15% 500K HES | 27.3 ± 2.0% | 74.7 ± 11.3 fl | 73.7 ± 9.6% |
| 10% 24K PVP 10% 500K HES | 28.1 ± 2.7% | 84.3 ± 8.1 fl | 67.8 ± 9.5% |
| 10% 24K PVP 5% 500K HES | 23.2% | 67.0 fl | 78.7% |

It can be seen that by using a mixture of polymers the 4-hr. whole blood stability of lyophilized reconstituted red cells is significantly improved over use of one polymer (PVP) alone.

## EXAMPLE 2

This example illustrates use of an automated blood bank cell washer. Packed red blood cells are mixed in a container with lyophilization buffer at a hematocrit of 30%. The lyophilization buffer is as described above, with the polymer mixture used containing 3% 360K PVP and 15% 500K HES.

The container is then placed in a standard shelf lyophilizer (Virtis SRC-15 Lyophilizer) and frozen. The frozen sample is then placed under a vacuum of 10-30 mtorr. The sample is allowed to dry, with a total weight loss of 58±2%. The sample is returned to room temperature and the vacuum is removed.

To reconstitute the dried samples, an equal volume of pre-warmed reconstitution buffer at 37°C is added to samples and swirled until sample is fully hydrated.

The reconstitution buffer is as described in Example 1.

The reconstituted sample is prediluted with an equal volume of reconstitution buffer and swirled until thoroughly mixed. The reconstituted and prediluted cells are transferred to a COBE 2991 Blood Cell Washer, centrifuged at 3000 rpm for 20 minutes, and repeated until all of the reconstitution buffer volume is added to the Cobe bag. The cells are washed by the automatic protocol of the Cell Washer with the following solutions described in Example 1:

1. Wash buffer: 500 ml, 1X, 3000 rpm, 20 minutes.
2. Pellets washed with Diluent buffer: 500 ml, 1X, 3000 rpm, 5 minutes.
3. Transfusion buffer: 500 ml, 4X, 3000 rpm, 5 minutes.

TABLE 2

| Sample No. | % Hb Recovery | MCV | % Whole Blood Stability |
|---|---|---|---|
| 1 | 27.3 | 80.0 | 73.3 |
| 2 | 26.2 | 76.1 | 73.3 |
| 3 | 29.6 | 78.7 | 62.5 |
| 4 | 27.2 | 80.5 | 70.9 |
| 5 | 29.4 | 76.1 | 70.6 |
| 6 | 24.7 | 76.1 | 71.7 |
| 7 | 26.5 | 80.0 | 68.2 |
| MEAN | 27.3±1.77 | 78.2±2.0$\mu m^3$ | 70.1±3.8 |

Note: MCV = mean cell volume

This example shows the use of the automated cell washing equipment with the disclosed centrifugation

conditions, to prepare reconstituted, washed human red cells.

## EXAMPLE 3

The procedure described in Example 1 was repeated with the substitution of 200K HES for 500K HES in a given HES/PVP polymer mixture in the lyophilization buffer. All other conditions were the same as those in Example 1. The results are described in Table 3. the use of 500K HES is marginally preferred over 200K HES in the polymer mixture.

TABLE 3

| Lyophilization Buffer Polymer Composition | Hemoglobin Recovery | Mean Cellular Volume | 4 hr. Whole Blood Stability |
|---|---|---|---|
| 5% 24K PVP 15% 200K HES | 14.7% | 77.3fl | 65.1% |
| 10% 24K PVP 10% 200K HES | 27.7 ± 4.4% | 81.8 ± 1.8fl | 61.6% |

## EXAMPLE 4

The procedure described in Example 1 was repeated with lyophilization buffers using 40% hematocrit mixtures with washed red blood cells. The polymer composition used in these lyophilization buffers, was 5:15% 24K PVP:500K HES. The glucose concentration in the 40% lyophilization buffers is increased to 2.3 M (441.37 g/l). All other conditions were the same as those in Example 1. The results are described as follows:

TABLE 4

| Sample Hct. | Lyophilization Buffer Polymer Composition | Hb Recovery | MCV | 4 hr. Whole Blood Stability |
|---|---|---|---|---|
| 40% | 20% 24K PVP (Control) | 28.2±3.5% | 80.0±7.9fl | 39.5±1.0% |
| 40% | 5% 24K PVP 15% 500K HES | 29.2±3.0% | 82.9±12.9fl | 70.1±14.8% |

The 4-hr. whole blood stability was significantly increased using a polymer mixture as compared to using a single polymer.

## EXAMPLE 5

The data shown in Table 5 indicate significant improvement in the osmotic stability, maximum cell deformability (DI max), and cell density in cells lyophilized with the buffers modified with various supplements. The osmotic stability assay was done with $^{51}Cr$ radiolabeled cells. Cell density was determined using discontinuous (step) density gradient centrifugation, which is a standard laboratory procedure. The method and equipment to measure the DI max is published in Mohandas, N., Clark, M.R., Health, B.P., Rossi, M., Wolfe, L.C., Lus, S.E., and Shohet, S.B. (1985) Blood 59, 768-774.

TABLE 5

| Parameter | Fresh Cells | 10 mM GSH (n = 4) | 40 mM GSH + 14% albumin (n = 1) | 10 mM GSH + 10 mM EDTA (n = 2) |
|---|---|---|---|---|
| Osmotic Stability (%) | 98-100 | 77.8 +/-3.2 | 75.4 | 78.0 +/-4.4 |
| MCV (fl) | 89.9 +/-3.4 (n = 56) | 73.4 +/-1.2 | 69.4 | 64.2 +/-6.6 |
| MCH (pg) | 30.7 +/-1.9 (n = 56) | 20.1 +/-1.6 | 17.6 | 18.3 +/-1.6 |
| MCHC (%) | 34.2 +/-1.5 (n = 56) | 27.3 +/-1.7 | 25.3 | 28.5 +/-0.35 |
| Final % OxyHb | 95-100 | 91.5 +/-7.4 | 95.4 | 96.9 +/-1.9 |
| Final % MetHb | 0-5 | 6.6 +/-7.2 | 4.6 | 2.1 +/-0.56 |
| Final % Hemichrome | 0-1 | 1.4 +/-1.0 | 0 | 1.0 +/-0.016 |
| DI (max) | 0.672 +/-0.06 (n = 29) | 0.375 +/-0.017 | 0.475 | 0.508 +/-0.016 |
| DI (max) as % of Fresh | 100 | 59.4 +/-4.0 | 73.3 | 73.7 +/-0.6 |
| Density (g/ml) | 1.10 | 1.083 +/-0.002 | 1.092 | 1.0835 +/-0.000 |

Note that the osmotic stability in the cells treated with the supplements is at least about 75% of fresh cells. Preferably, by use of the invention osmotic stability is at least 60% of the stability of whole blood, and the DI(max) is at least 50% of the DI(max) measured with fresh red cells.

Notes:

1) Osmotic stability of $^{51}Cr$ labeled red cells suspended in physiological saline at room temperature.
2) MCV is the mean corpuscular volume in femtoliters.
3) MCH is the mean corpuscular hemoglobin in picograms.
4) MCHC is the mean corpuscular hemoglobin concentration as a w/v percent.
5) OxyHb is functional oxyhemoglobin measured as a percent recovery at the final stage (cells washed into transfusion buffer).
6) MetHb is oxidized methemoglobin (again % recovery at final step).
7) Hemichrome is a class of several forms of irreversibly degraded hemoglobin (% recovery at final step).
8) DI (max) is a measure of the maximum deformability (ellipticity) of red cells subjected to mechanical shear stress.
9) Small changes in cell density reflect significant changes in overall cell quality and morphology.
10) GSH is reduced glutathione.
11) EDTA is sodium ethylenediamine tetraacetate.
12) Albumin is serum albumin prepared from human plasma or bovine plasma.
13) Other antioxidants in addition to GSH include alpha-tocopherol used at 1-3 mg/gram of red cells.
14) Other chelators besides EDTA include desferrioxamine used at 1-10 mM.
15) All data obtained using human red blood cells.

## EXAMPLE 6

In the following Tables 6 and 7, one particular advantage of including albumin in the lyophilization buffer is shown (the experiment of Table 7 is the same as the 40 mM GSH + 14% albumin column in Table 5) in terms of a dramatic improvement in the cell density profile.

Table 6 and 7 show the fraction of lyophilized reconstituted human red cells that sediment above or below a solution (the density step gradient "cushion") of a known solution density. The percent of cells below the density cushion (i.e., having a cell density greater than the solution density) is indicated. The same percentage profile for normal human red cells as a control is also shown.

The lyophilization buffer was as described in Example 1, supplemented with GSH or GSH/albumin. One can see that the human red cells lyophilized in the above lyophilization buffer containing GSH and albumin supplements is shifted to near normal, which is also

reflected by the high average cell density (1.092 g/ml as shown in Table 5). Such a population of cells with near-normal density can be expected to have excellent cell morphology, with reduced damage due to processing, and minimal cell-cell aggregation. Comparable tests using an antioxidant such as GSH alone do not yield such high cell density (1.083 +/- 0.002 g/ml as shown in Table 5, or 1.086 using 40 mM GSH alone as shown in Table 6). One can appreciate from the data that small differences in cell density translate into significant improvements in cell quality, with minimal cell-cell aggregates.

TABLE 6

40 mM GSH Lyo. Buffer

| Density Gradient Separation | | | | |
|---|---|---|---|---|
| Density | Above | Below | % | Norm |
| 1.046 | 0.5 | 37.0 | 98.7 | 100.0 |
| 1.054 | 1.0 | 40.0 | 97.6 | 100.0 |
| 1.062 | 4.5 | 36.0 | 89.9 | 99.7 |
| 1.066 | 5.0 | 33.0 | 86.8 | 99.4 |
| 1.078 | 11.0 | 27.0 | 71.1 | 99.1 |
| 1.086 | 14.0 | 14.0 | 50.0 | 97.2 |
| 1.096 | 19.5 | 9.0 | 31.6 | 96.0 |
| 1.094 | 22.5 | 5.0 | 18.2 | 90.0 |
| 1.102 | 34.0 | 1.0 | 2.9 | 35.3 |
| 1.110 | 33.5 | 0.0 | 0.0 | 5.6 |

TABLE 7

40 mM GSH + 14% w/v Albumin Lyo. Buffer

| Sample No.: 91-0670 Density Gradient Separation | | | | |
|---|---|---|---|---|
| Density | Above | Below | % | Norm |
| 1.046 | 3.0 | 43.0 | 93.3 | 100.0 |
| 1.054 | 3.5 | 46.5 | 93.0 | 100.0 |
| 1.062 | 6.0 | 33.0 | 84.6 | 99.7 |
| 1.066 | 8.0 | 31.5 | 79.7 | 99.4 |
| 1.078 | 11.5 | 30.5 | 72.6 | 99.1 |
| 1.086 | 14.0 | 27.0 | 65.9 | 97.2 |
| 1.090 | 19.0 | 24.0 | 55.8 | 96.0 |
| 1.094 | 25.0 | 17.0 | 40.5 | 90.0 |
| 1.102 | 31.0 | 4.0 | 11.4 | 35.3 |
| 1.110 | 40.0 | 0.5 | 1.2 | 5.6 |

EXAMPLE 7

Blood was obtained from six healthy adult individuals with no history of either hemoglobinopathy or

abnormal RBC metabolism. Blood was withdrawn from each donor into plastic transfer bags (Fenwal Laboratories, Deerfield, Ill) containing 63mL of citrate phosphate dextrose-adenine (CPD-A) anticoagulant using conventional blood banking techniques. The blood units (500ml each) were centrifuged at 1500g for 5 minutes at room temperature (22C) to remove the buffy coat and plasma. The packed RBC were washed in isotonic dextrose saline according to standard washing procedures [11] using automatic cell washer (Model 2991, COBE, Lakewood, CO). The washed and packed RBC (about 85% hematocrit) were resuspended to about 40% in lyophilization buffer as described in Example 2. (1800mOsmol, pH 7.4). About 360g of the RBC suspension were transferred to plastic lyophilization bags and were placed in a conventional pharmaceutical shelf freeze-dryer (Cryopharm Corporation, Pasadena, CA) and then freeze-dried as described in Example 2. At the end of the lyophilization cycle, the dried RBC were rehydrated and reconstituted in phosphate buffered rehydration buffers described in Example 2 (360mOsmol, pH 7.4) at 22C. Briefly, to rehydrate the RBC, 600g of rehydration buffer was added to the dried RBC and then agitated on a wrist action shaker (Burrel Corporation, Pittsburgh, PA) until the RBC were fully rehydrated. At the end of the rehydration, additional 600g of rehydration buffer was added to the sample and then centrifuged at 1500g for 3 minutes. The supernatant was removed and the packed RBC were washed twice in wash buffers as described in Example 2 by centrifugation at 1500g, using COBE automatic cell washer. Reconstituted RBC were assayed for glycolytic enzyme activities and intermediates according to published methods.

Control blood samples were obtained from autologous donors at the time of reconstitution of lyophilized RBC. Control RBC were treated similarly to reconstituted lyophilized RBC with respect to washing. In addition the glycolytic enzyme activities of blood bank stored RBC were determined. See Tables 1 and 2.

Rate of Adenine Nucleotide Synthesis: The rate of adenine nucleotide synthesis was measured by following the incorporation of carbon 14-labelled adenine into the adenine nucleotide pool in intact RBC according to the method described by Zerez et al. J. Lab. Clin. Med. 114, 43-50 (1989). Briefly, the RBC were incubated with carbon 14-labelled adenine ($^{14}C$) at 37C and at different times aliquots were removed, mixed with saline and immediately immersed in boiling water for 60 seconds. The mixture was chilled at 0°C and then centrifuged to remove coagulated proteins. The resultant supernatant contained $^{14}C$-labelled adenine nucleotides along with an excess of $^{14}C$-labelled adenine. A modification of the method of Hershko [19] was used to separate $^{14}C$-labelled adenine nucleotides from $^{14}C$-adenine and radioactivity was counted in a liquid scintillation spectrometer (Model LS7500, Beckman instruments, Fullerton, CA).

The rate of Methemoglobin Reduction: The rate of methemoglobin (metHb) reduction in intact RBC was determined by using a published method. Zerez et al. Blood 76, 1008-1014 (1990). Briefly, to convert hemoglobin (Hb) to metHb, washed RBC were incubated for 10 minutes at 37C in a solution containing 0.1% (wt/v) $NaNO_3$, 605mM $Na_6HPO_4$, pH 7.4 and 154mM NaCl at

final packed cell volume of 25%. This resulted in 95-100% of conversion of Hb to metHb. To remove $NaNO_3$ RBC were washed 6 times with 5 volumes of isotonic saline. The washed RBC were resuspended in phosphate buffered saline containing 10mM D-glucose and incubated at 37C. Aliquots were withdrawn at different intervals. The percentage of methemoglobin remaining was measured spectrophotometrically. Hegesh et al. Clin. Chim. Acta 30, 679-682 (1970). The rate of methemoglobin repair, presumably by conversion to oxyhemoglobin, was estimated as described by Zerez et al. See FIG. 1.

Other methods: Rates of ATP and lactate production were determined by the methods described by Beutler, Red Cell Metabolism: A Manual of Biochemical Methods, Beutler, E., Ed., Grune & Stratton, 2nd Ed., pp. 122-146 (1984).

Statistical Analysis: Differences between lyophilized and non-lyophilized RBC were analyzed with two tailed Student's t-test for paired data. Comparison between lyophilized and blood bank stored RBC were made using two tailed Student's t-test for independent data. See FIG. 2.

Table 1. Summary of the activities of the glycolytic enzymes in hemolysates from rehydrated lyophilized and non-lyophilized RBC.

Enzyme activity, umol/min/ g Hb

| Enzyme | Lyo | N-lyo | BB | N-R | P |
|---|---|---|---|---|---|
| HX* | 1.26±0.22 | 1.65±0.10 | 1.20±0.12 | 0.98-1.3 | NS |
| PGI* | 44.7±4.57 | 44.3±2.66 | 48.3±6.03 | 43.7-65.8 | NS |
| PFK* | 12.1±1.61 | 11.7±0.97 | 9.73±2.18 | 8.44-12.2 | NS |
| Ald* | 3.59±0.41 | 3.72±0.54 | 2.39±0.34 | 1.97±3.59 | NS |
| TPI* | 1750±460 | 2140±490 | 2900±777 | 2130-3340 | P<0.005 |
| G3PD* | 318±68.4 | 311±43.0 | 244±72.0 | 238-346 | NS |
| DPGM* | 5.34±0.72 | 4.64±0.91 | 8.43±2.23 | 8.43±2.23 | P<0.015 |
| PGK* | 340±147 | 340±115 | 349±47.7 | 212-341 | NS |
| PGM* | 35.2±5.09 | 38.1±5.99 | 17.3±6.70 | 13.9-38.0 | NS |
| Eno* | 4.99±0.99 | 7.60±0.87 | 4.96±0.89 | 4.2-6.58 | p<0.001 |
| PK* | 18.9±5.71 | 21.1±5.40 | 15.0±2.14 | 12.5-17.2 | p<0.032 |
| LDH* | 231±29.0 | 190±19.2 | 141±56.4 | 145-203 | p<0.001 |
| G6PD+ | 12.4±1.55 | 14.7±1.82 | ND | 9.90-13.2 | NS |
| 6PGD+ | 11.1±0.99 | 10.0±1.09 | ND | 7.27-10.0 | NS |
| TA+ | 0.97±0.21 | 1.10±0.34 | ND | 0.78-1.32 | NS |
| TK+ | 0.68±0.13 | 0.93±0.66 | ND | 0.50-1.03 | NS |

Data represent the mean ± sd, for 6 samples. Data from blood bank stored RBC are included for comparison with rehydrated lyophilized RBC. Total number of blood bank samples analyzed was 3. Abbreviations: lyo, lyophilized; N-lyo, non-lyophilized; BB, Blood bank,; N-R, normal range; P, probability for comparison between lyophilized and non-lyophilized RBC; ND, not detected; NS, not significant.* Enzymes of Glycolytic Pathway; + Enzymes of the Pentose Phosphate Pathway.

The preferred useful reconstituted RBCs are characterized by hexokinase (HX) activity of at least 0.9 micromole/min/gram hemoglobin; diphosphoglyceromutase (DPGM) activity of at least

3.0 micromole/min/gm hemoglobin; phosphofructokinase (PFK) activity of at least 8.0 micromole/min/gram hemoglobin; pyruvate kinase (PK) activity if at least 12.0 micromole/min/gm hemoglobin; glucose-6-phosphate dehydrogenase (G-6-PD) of at least 9.0 micromole/min/gm hemoglobin; 6-phosphogluconate dehydrogenase (6-PGD) of at least 7.0 micromole/min/gm hemoglobin; at least 0.5 micromole/min/gm hemoglobin each of transaldolase (TA) and transketolase (TK); and at least 6.0 micromole/min/gm hemoglobin of glutathione reductase.

Table 2. Comparison of the levels of glycolytic intermediates in rehydrated lyophilized and fresh non-lyophilized RBC.

Concentrations of intermediates, nmols/ g Hb

| Intermediates | Lyo | N-lyo | NV | P |
|---|---|---|---|---|
| G6P | 49.8±72.1 | 76.5±102 | 100±28.0 | NS |
| F6P | 0.92±2.26 | 3.05±7.47 | 15.6±6.30 | NS |
| FDP | 7.60±425 | 1.49±179 | 4.70±1.60 | NS |
| DHAP | 1770±887 | 174±147 | 37.5±3.10 | p<0.012 |
| GAP | 112±46.8 | 44.9±43.5 | 9.38±6.30 | NS |
| 2,3-DPG | 3152±938 | 9633±2640 | 13500±2000 | p<0.004 |
| 3PG | 611±210 | 134±56.1 | 122±28.0 | p<0.006 |
| 2PG | 338±252 | 216±165 | 31.3±13.0 | p<0.046 |
| PEP | 216±104 | 67.5±50.8 | 50.0±16.0 | p<0.01 |
| Pyr | 170±52.2 | 193±125 | 84.4±25.0 | NS |
| Lact | 6032±2730 | 9495±3542 | 1140±370 | NS |
| ATP | 1758±392 | 3875±780 | 3220±280 | p<0.008 |
| ADP | 1743±316 | 700±133 | 409±56.0 | p<0.003 |
| AMP | 2370±343 | 204±125 | 134±25.0 | p<0.001 |

Data represent the mean ± S.D. for 6 samples. Normal values are included in the table for comparison with present data. Abbreviations: lyo, lyophilized; N-lyo, non-lyophilized; NV, normal values; P, probability for comparisons between lyophilized and non-lyophilized RBC.

The preferred useful reconstituted RBCs are characterized by at least 50 nmole/gm hemoglobin of glucose-6-phosphate (G6P); at least 100 nmole/gm hemoglobin of fructose-1,6-diphosphate (FDP); at least 2000 nmole/gm hemoglobin of 2,3-diphosphoglycerate (2,3-DPG); and at least 50 nmole/gm hemoglobin of pyruvate (pyr).

The foregoing data provides evidence that human red cells lyophilized and reconstituted by the process of the invention retain the ability to reduce methemoglobin (nonfunctional) to the physiological and oxygen-carrying state, and to preserve key glycolytic enzyme activities at levels comparable to non-lyophilized red cells or refrigerated red cells stored by current methods. Key enzymes include hexokinase (HX) which has the lowest activity in normal cells, hence is thought to be the rate-limiting step in the pathway; and phosphofructokinase (PFK) and pyruvate kinase (PK), whose reactions involve the largest calculated free energy changes between substrate and product.

The reconstituted lyophilized red cells retain the activity of diphosphoglyceromutase, which in human red cells shunts, 1,3-diphosphoglycerate (1,3-DPG), a glycolytic intermediate, to 2,3-DPG, which is a key allosteric effector of hemoglobin, and regulates the

ability of hemoglobin to bind and deliver oxygen. The data shows steady-state levels of the metabolic intermediates to include levels of glucose-6-phosphate (G6P), the product of hexokinase activity; fructose-1,6-diphosphate (FDP), the product of phosphofructokinase activity; 2,3-DPG, the product of diphosphoglyceromutase activity; and pyruvate (pyr), the product of pyruvate kinase (PK) activity. Furthermore, the enzymes of the pentose phosphate shunt are functional; this pathway serves two vital functions in the red cell: it produces energy (ATP) and ribose-5-phosphate (R-5-P) used to make reduced glutathione as part of the cell's normal antioxidant defense system, and it produces 5-phosphoribosyl pyrophosphate (PRPP), an intermediate used to make adenine nucleotides from exogenous adenine (exogenous adenine is imported into the cell from plasma, or in refrigerated stored cells from commercial storage solutions such as CPDA-1: citrate/phosphate/dextrose/adenine). Finally, the data suggests key high energy intermediates such as reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) can be made via the normal glycolytic pathway in the reconstituted cells and these reduced dinucleotides are key cofactors for the enzymes methemoglobin reductase (NADH) and glutathione reductase (NADPH).

From the foregoing description, one skilled in the art can readily ascertain the essential characteristics of the invention and, without departing from the spirit and scope thereof, can adapt the invention to various usages and conditions. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render

expedient, and although specific terms have been employed herein they are intended in a descriptive sense and not for purposes of limitation.

WHAT IS CLAIMED IS:

1. A process for the lyophilization of cells or cell-like materials, comprising:

immersing a plurality of cells in a buffered solution which includes:

a monosaccharide which is present in the solution in a concentration of from about 7.0 to 37.5%, and

a mixture comprising at least two different polymers, each of said polymers having a number average molecular weight in the range of about IK to about 600K, wherein the total concentration of said polymers is of from about 0.7% up to saturation in the solution; freezing the solution; and

drying the cells by sublimation of the water.

2. The process of Claim 1 wherein said polymers are amphipathic.

3. The process of Claim I wherein one of said polymer has a molecular weight in the range of about 20K to about 360K and another of said polymers has a molecular weight in the range of about 100K to 500K.

4. The process of Claim 1 wherein the monosaccharide is selected from the group consisting of pentoses and hexoses.

5. The process of Claim 4 wherein the monosaccharide is selected from the group consisting of xylose, glucose, ribose, mannose and fructose.

6. The process of Claim 3 wherein said mixture of polymers comprises polyvinylpyrrolidone and hydroxyethyl starch.

7.The process according to Claim 1 wherein said buffered solution further comprises an antioxidant, chelating agent, protein, or mixtures thereof.

8. A process according to Claim 7 wherein said antioxidant comprises glutathione.

9. A process according to Claim 7 wherein said antioxidant comprises alpha-tocopherol.

10. A process according to Claim 7 wherein said chelating agent comprises EDTA.

11. A process according to Claim 7 wherein said chelating agent comprises desferrioxamine.

12. A process according to Claim 7 wherein said protein comprises bovine serum albumin.

13. A process according to Claim 7 wherein said protein comprises human serum albumin.

14. A medium for the lyophilization of cells, comprising:

a buffered solution containing:

a monosaccharide which is present in the solution in a concentration of from about 7.0 to 37.5%, and

a mixture comprising at least two different polymers, each of said polymers having a molecular weight of from about 1K to about 600K,

wherein the total which is present in a concentration of said polymers is from about 0.7% up to saturation of the solution.

15. A medium according to Claim 14 wherein said polymers are amphipathic.

16. A medium according to Claim 14 wherein one of said polymers has a molecular weight in the range of about 20K to about 360K and another of said polymers has a molecular weight in the range of about 100K to 500K.

17. The medium of Claim 14, 15, or 16 wherein the monosaccharide is selected from the group consisting of pentoses and hexoses.

18. The medium of Claim 17 wherein the monosaccharide is selected from the group consisting of xylose, glucose, ribose, mannose and fructose.

19.The medium of Claim 18 wherein said mixture of polymers comprises polyvinylpyrrolidone and hydroxyethyl starch.

20. A medium according to Claim 19 wherein said polyvinylpyrrolidone has a molecular weight of about 24K and said hydroxyethyl starch has a molecular weight of about 500K.

21. A medium according to Claim 19 wherein said polyvinylpyrrolidone has a molecular weight of about 24K and said hydroxyethyl starch has a molecular weight of about 200K.

22. A medium according to Claim 19 wherein said polyvinylpyrrolidone has a molecular weight of about 360K and said hydroxyethyl starch has a molecular weight of about 500K.

23. A medium according to Claim 14 further comprising an antioxidant, chelating agent or protein.

24. A medium according to Claim 23 wherein said antioxidant comprises glutathione.

25. A medium according to Claim 23 wherein said antioxidant comprises alpha-tocopherol.

26. A medium according to Claim 23 wherein said chelating agent comprises EDTA.

27. A medium according to Claim 23 wherein said medium comprises desferrioxamine.

28. A medium according to Claim 23 wherein said protein comprises bovine serum albumin.

29. A medium according to Claim 23 wherein said protein comprises human serum albumin.

30. A medium for reconstituting lyophilized blood cells, comprising:

a buffered solution containing a polymer having a number average molecular weight in the range of about 1K to 600K.

31. A medium according to Claim 30 wherein said polymer is amphipathic.

32. A medium according to Claim 31 wherein said molecular weight is in the range of 1K to 360K.

33. A medium according to Claim 32 wherein said polymer comprises polyvinylpyrrolidone.

34. A medium according to Claim 33 wherein said polymer is present in a concentration range of 1 to 20 weight by volume %.

35. A medium according to Claim 34 comprising about 10% 24K polyvinylpyrrolidone.

36. A medium according to Claim 34 comprising about 19.0% 10K polyvinylpyrrolidone.

37. A medium according to Claim 35 or 36 comprising about 1.47 mM $KH_2PO_4$, about 100.7 mM NaCl, and about 8.1 mM $Na_2HPO_4$.

38. A medium according to Claim 30 further comprising an antioxidant, chelating agent or protein.

39. A medium according to Claim 38 wherein said antioxidant comprises glutathione.

40. A medium according to Claim 38 wherein said antioxidant comprises alpha-tocopherol.

41. A medium according to Claim 38 wherein said chelating agent comprises EDTA.

42. A medium according to Claim 38 wherein said chelating agent comprises desferrioxamine.

43. A medium according to Claim 38 wherein said protein comprises bovine serum albumin.

44. A medium according to Claim 38 wherein said medium comprises human serum albumin.

45. A medium for washing reconstituted blood cells, comprising:

a buffered solution containing a polymer having a number average molecular weight in the range of about 1K to 600K; inosine; adenine, nicotinic acid, glutamine, and a monosaccharide.

46. A medium according to Claim 45 wherein said polymer is amphipathic.

47. A medium according to Claim 46 wherein said molecular weight is in the range of 1K to 360K.

48. A medium according to Claim 47 wherein said polymer comprises polyvinylpyrrolidone.

49. A medium according to Claim 47 wherein said polymer is present in a concentration range of 1 to 20 weight %.

50. A medium according to Claim 49 wherein said monosaccharide is selected from the group consisting of pentoses and hexoses.

51. A medium according to Claim 50 wherein said monosaccharide is selected from the group consisting of xylose, glucose, ribose, mannose and fructose.

52. A medium according to Claim 51 comprising about 16% 24K polyvinylpyrrolidone.

53. A medium according to Claim 51 comprising about 16% 40K polyvinylpyrrolidone.

54. A medium according to Claim 52 comprising about 10.0 mM Inosine, 5.0 mM Adenine, 0.75 mM Nicotinic acid, 0.75 mM Glutamine, 0.49 mM $MgCl_2$ $6H_2O$, 5.0 mM KCl, 75.0 mM NaCl, 10.3 mM $Na_2HPO_4$ and 20.0 mM Glucose.

55. A medium according to Claim 54 comprising 10 mM Inosine, 5 mM Adenine, 0.75 mM Nicotinic acid, 0.75 mM Glutamine, 0.49 mM $McGl_2$ $6H_2O$, 30.0 mM KCl, 30.0 mM NaCl, 10.0 mM $Na_2HPO_4$,$\cdot 7H_2O$, and 20 mM Glucose.

56. A medium according to Claim 45 further comprising an antioxidant, chelating agent, protein or mixtures thereof.

57. A medium according to Claim 56 wherein said antioxidant comprises glutathione.

58. A medium according to Claim 56 wherein said medium comprises alpha-tocopherol.

59. A medium according to Claim 56 wherein said chelating agent comprises EDTA.

60. A medium according to Claim 56 wherein said chelating agent comprises desferrioxamine.

61. A medium according to Claim 56 wherein said protein comprises bovine serum albumin.

62. A medium according to Claim 56 wherein said protein comprises human serum albumin.

63. A medium for resuspending a washed blood cell comprising a buffered solution containing sodium pyrophosphate, KCl, $KH_2PO_4$, $Na_2HPO_4$ and ATP.

64. A medium according to Claim 63 comprising about 61.1 mM sodium pyrophosphate, 1.19 mM KCl, 0.88 mM $KH_2PO_4$, 11.1 mM NaCl, 4.86 mM $Na_2HPO_4$, 8.89 mM ATP.

65. A medium for suspending blood cells for transfusion comprising a buffered solution containing a polymer having a number average molecular weight in the range of from about 1K to 600K.

66. A medium according to Claim 65 wherein said polymer is amphipathic.

67. A medium according to Claim 66 wherein said molecular weight is in the range of 1 to 10K.

68. A medium according to Claim 67 wherein said polymer comprises polyvinylpyrrolidone.

69. A medium according to Claim 68 wherein said polymer is present in a concentration range of 1 to 20 weight %.

70. A medium according to Claim 69 further comprising a monosaccharide selected from the group consisting of pentoses and hexoses.

71. A medium according to Claim 70 wherein said monosaccharide is selected from the group consisting of xylose, glucose, ribose, mannose and fructose.

72. A medium according to Claim 71 comprising about 10% 2.5K polyvinylpyrrolidone.

73. A medium according to Claim 72 comprising about 68.4 mM NaCl, 5.0 mM $Na_2HPO_4$, 10.0 mM Glucose.

74. A process according to Claim 1 further comprising the step of reconstituting the dried cells in a buffered solution containing a polymer having a number average molecular weight in the range of about 1K to 600K.

75. A process according to Claim 74 further comprising the step of washing the reconstituted cells in a buffered wash solution containing a polymer having a number average molecular weight in the range of about 1K to 600K; inosine; adenine; nicotinic acid; glutamine and a monosaccharide.

76. A process according to any one of Claims 1 through 6, 74 or 75 wherein said cells comprise erythrocytes.

77. A process according to any one of Claims 1 through 6, 74 or 75 wherein said cells comprise platelets.

78. A process according to any one of Claims 1 through 6, 74 or 75 wherein said cells comprise cells cultured in vitro.

79. A process according to any one of Claims 1 through 6, 74 or 75 wherein said cells comprise peripheral blood cells.

80. A process according to any one of Claims 1 through 6, 74 or 75 wherein said cells comprises stem cells.

81. A process according to any one of Claims 1 through 6, 74 or 75 wherein said cell-like material comprises liposomes.

82. A process according to any one of Claims 1 through 6, 74 or 75 wherein said cell-like material comprises hemosomes.

83. A process according to any one of Claims 1 through 6, 74 or 75 wherein said cell-like material comprises cell membrane ghost preparations.

84. A process according to Claim 78 wherein said cultured cells comprised mammalian cells.

85. A process according to Claim 84 wherein said mammalian cultured cells comprises hybridoma cells.

86. A lyophilized reconstituted blood cell composition having an osmotic stability in whole blood of at least 60%.

87. A lyophilized reconstituted red blood cell composition having a DI(max) that is at least 50% of the DI(max) measured with fresh red cells.

88. A lyophilized reconstituted red blood cell composition having an average cell density of at least 1.083 ± 0.002 grams/ml.

89. A composition according to any one of Claims 86 through 86 wherein said red blood cells comprise human red blood cells.

90. A transfusibly useful red blood cell composition wherein said red blood cells are characterized by at least 60% osmotic stability in whole blood, DI(max) at least 50% of fresh red blood cells, and average cell density at least 1.083 ± 0.002 g/ml.

91. A product prepared by any one the processes of Claims 1 through 13.

92. A product according to the process of Claim 76.

93. A product according to the process of Claim 77.

94. A product according to the process of Claim 78.

95. A product according to the process of Claim 79.

96. A product according to the process of Claim 80.

97. A product according to the process of Claim 81.

98. A product according to the process of Claim 82.

99. A product according to the process of Claim 83.

100. A product according to the process of Claim 84.

101. A product according to the process of Claim 85.

102. A process according to Claim 76 wherein said erythrocytes retain a methemoglobin repair half-life of less than 30 hours.

103. A process according to Claim 76 wherein said erythrocytes maintain physiological activity levels of enzymes of the glycolytic pathway.

104. A process according to Claim 103 wherein said enzyme activity comprises hexokinase (HX) activity of at least 0.9 micromole/min/gram hemoglobin.

105. A process according to Claim 103 wherein said enzyme activity comprises diphosphoglyceromutase (DPGM) activity of at least 3.0 micromole/min/gram hemoglobin.

106. A process according to Claim 103 wherein said enzyme activity comprises phosphofructokinase (PFK) activity of at least 8.0 micromole/min/gram hemoglobin.

107. A process according to Claim 103 wherein said enzyme activity comprises pyruvate kinase (PK) activity of at least 12.0 micromole/min/gram hemoglobin.

108. A process according to Claim 76 wherein said erythrocytes comprise physiological levels of glycolytic chemical intermediates.

109. A process according to Claim 108 wherein said chemical intermediates comprise glucose-6-phosphate (G6P) of at least 50 nmole/gram hemoglobin.

110. A process according to Claim 108 wherein said chemical intermediates comprise fructose-1,6-diphosphate (FDP) of at least 100 nmole/gram hemoglobin.

111. A process according to Claim 108 wherein said chemical intermediates comprise 2,3-diphosphoglycerate (2,3-DPG) of at least 2000 nmole/gram hemoglobin.

112. A process according to Claim 108 wherein said chemical intermediates comprise pyruvate (pyr) of at least 50 nmole/gram hemoglobin.

113. A process according to Claim 76 wherein said erythrocytes comprise physiological activity levels of enzymes of the pentose phosphate shunt.

114. A process according to Claim 113 wherein said enzymes comprise glucose-6-phosphate dehydrogenase (G-6-PD) of at least 9.0 micromole/min/gram hemoglobin.

115. A process according to Claim 113 wherein said enzymes comprise 6-phosphogluconate dehydrogenase (6-PGD) of at least 7.0 micromole/min/gram hemoglobin.

116. A process according to Claim 113 wherein said enzymes comprise transaldolase (TA) of at lest 0.5 micromole/min/gram hemoglobin.

117. A process according to Claim 113 wherein said enzymes comprise transketolase (TK) of at least 0.5 micromole/min/gram hemoglobin.

118. A process according to Claim 76 wherein said erythrocytes comprise physiological activity levels of the enzyme glutathione reductase of at least 6.0 micromole/min/gram hemoglobin.

119. A process according to any of Claims 102 through 118 wherein said erythrocytes comprise human erythrocytes.

120. A product according to the process of Claim 102.

121. A product according to the process of Claim 103.

122. A product according to the process of Claim 104.

123. A product according to the process of Claim 105.

124. A product according to the process of Claim 106.

125. A product according to the process of Claim 107.

126. A product according to the process of Claim 108.

127. A product according to the process of Claim 109.

128. A product according to the process of Claim 110.

129. A product according to the process of Claim 111.

130. A product according to the process of Claim 112.

131. A product according to the process of Claim 113.

132. A product according to the process of Claim 114.

133. A product according to the process of Claim 115.

134. A product according to the process of Claim 116.

135. A product according to the process of Claim 117.

136. A product according to the process of Claim 118.

137. A product according to the process of Claim 119.

138. A lyophilized reconstituted red blood cell composition having physiological activity levels of enzymes that comprise the glycolytic pathway.

139. A lyophilized reconstituted red blood cell composition having a physiological half life for methemoglobin repair.

140. A lyophilized reconstituted red blood cell composition having physiological activity levels of glutathione reductase.

141. A lyophilized reconstituted red blood cell composition having physiological activity levels of enzymes that comprise the pentose phosphate shunt.

142. A lyophilized reconstituted red blood cell composition having physiological levels of chemical intermediates that comprise the glycolytic pathway.

143. A composition according to any one of Claims 138 through 142 wherein said red blood cells comprise human red blood cells.

144. A transfusibly useful red blood cell composition wherein said red blood cells are characterized by physiological activity levels of enzymes that comprise the glycolytic and pentose shunt pathways, physiological activity levels of glutathione reductase, physiological half life of methemoglobin repair, and physiological levels of glycolytic chemical intermediates.

145. A transfusibly useful red blood cell composition as in Claim 144 wherein said red blood cells comprise human red blood cells.

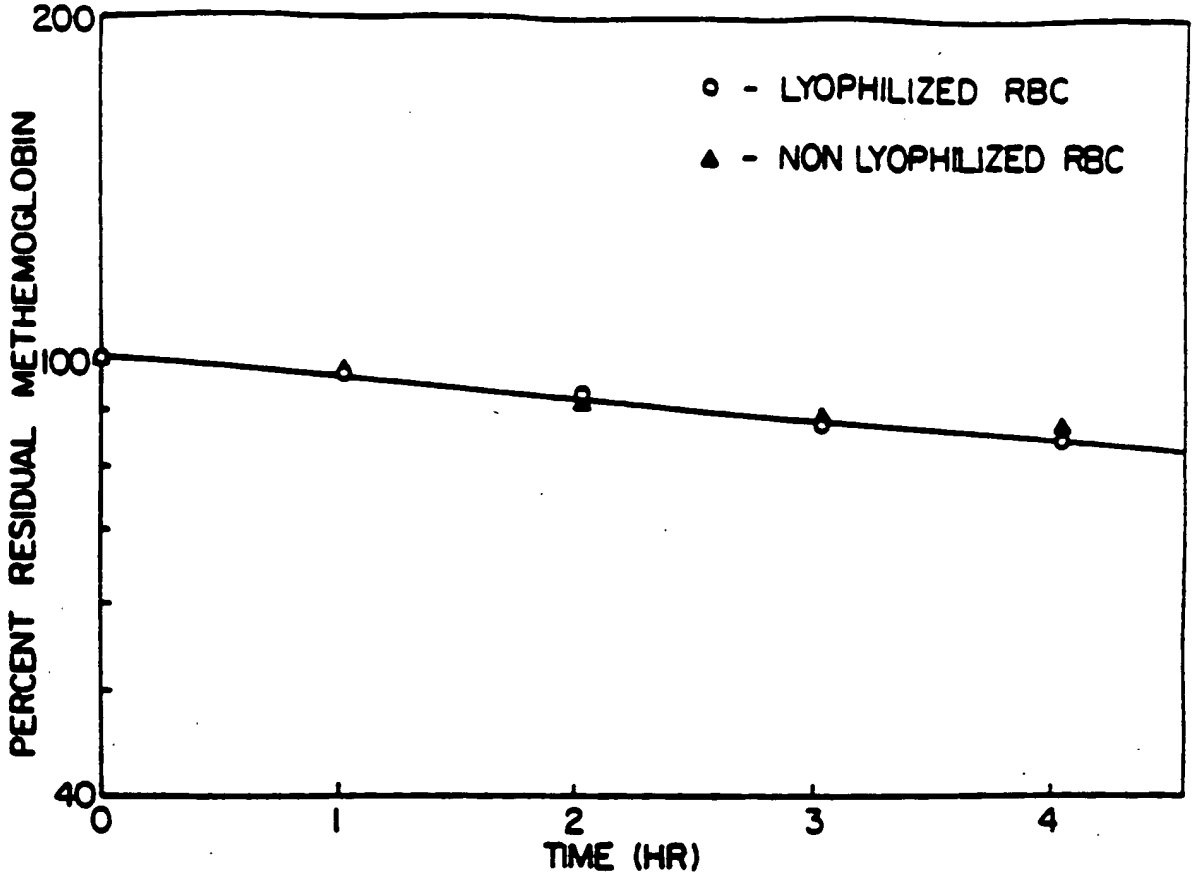
200
100
40
0
1
2
3
4
PERCENT RESIDUAL METHEMOGLOBIN
TIME (HR)
o - LYOPHILIZED RBC
▲ - NON LYOPHILIZED RBC

FIG. 1.

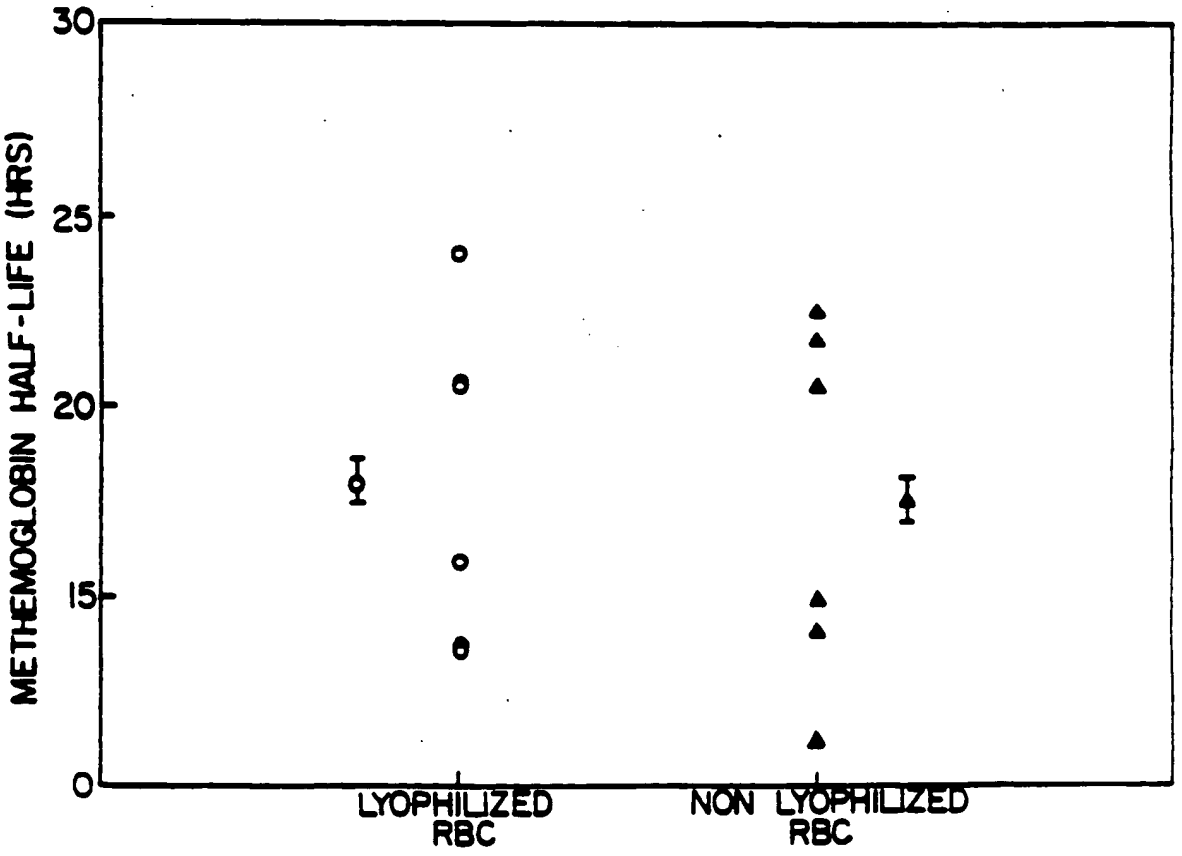
30
25
20
15
0
METHEMOGLOBIN HALF-LIFE (HRS)
LYOPHILIZED RBC
NON LYOPHILIZED RBC

FIG 2

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/03544

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A01N 1/02

U.S. CL.: 435/2

II FIELDS SEARCHED

Minimum Documentation Searched [7]

| Classification System | Classification Symbols |
|---|---|
| U.S. | 435/2 |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

| Category [10] | Citation of Document, [11] with indication, where appropriate, of the relevant passages [12] | Relevant to Claim No. [13] |
|---|---|---|
| X<br>Y | US, A, 4,874,690 (GOODRICH) 17 October 1989, see see entire document. | 1-13<br>14-85, 102-119 |
| Y | US, A, 3,987,159 (SPONA) 19 October 1976, see col. 4, lines 27-29. | 16, 19-21, 33-36, 48, 68 |
| Y | US, A, 3,915,794 (ZYGRAICH) 28 October 1975, see col. 1, lines 54-68. | 6,16, 19-21 31-36, 46-49 65-68 |
| Y,P | US, A, 4,963,362 (RAHMAN ET AL.) 16 October 1990, see entire document. | 81 |

* Special categories of cited documents: [14]

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 30 August 1990 | 11 SEP 1991 |

| International Searching Authority | Signature of Authorized Officer |
|---|---|
| ISA/US | L. Blaine Lankford (vsh) |

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